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(54) **SELECTION SYSTEM FOR EVOLVING PROTEASES AND PROTEASE-CLEAVAGE SITES**

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(57) **ABSTRACT**

The present invention relates to a fusion protein, comprising the structure N-PCS$^Y$-degSig$_N$-M-PCS$^X$-degSig$_C$-C; wherein N represents the N-terminus; PCS$^Y$ and PCS$^X$ each represent a protease cleavage site (PCS), which differ from each other in at least one amino acid residue; degSig$_N$ represents a degradation signal which promotes degradation of the fusion protein in a host pot cell if PCS$^Y$ is cleaved by a protease such that the first amino acid of degSig$_N$ becomes the new N-terminus of the remaining fusion; M represents a cytoplasmic selection marker; and degSig$_C$ represents a second degradation signal which promotes degradation of the fusion protein in a host cell if PCS$^X$ is not cleaved by a protease; and C represents the C-terminus. Further provided is a nucleic acid construct, comprising a nucleic acid sequence coding for said fusion protein, a nucleic acid expression construct library, comprising a plurality of such nucleic acid expression constructs in diversified form, and methods using the fusion protein and nucleic acid constructs coding therefor. Finally, the present invention provides variants of bdSUMO and bdSENP1 which have been identified by the methods of the present disclosure, and which exhibit improved properties over existing orthogonal protease/protease cleavage site-pairs which are currently used with wild-type bdSUMO and wildtype bdSENP1.

**Specification includes a Sequence Listing.**

A)
Elements of the selection system

Degron$^{NER}$ ⬤────● bdSUMO
ssrA ████────◆ bdSUMO*
bdSENP1 ═════ HygB

B)
bdSUMO-HygB-bdSUMO* + bdSENP1
(No degradation signals)

Bacterial survival
1:50 1:500 1:5000 1:50000 1:500000

C)
bdSUMO*-degron$^{NER}$-HygB-bdSUMO-ssrA + bdSENP1

Bacterial survival
1:50 1:500 1:5000 1:50000 1:500000

D)
bdSUMO-degron$^{NER}$-HygB-bdSUMO-ssrA + bdSENP1

HygB degradation

Bacterial death
1:50 1:500 1:5000 1:50000 1:500000

E)
bdSUMO*-degron$^{NER}$-HygB-bdSUMO*-ssrA + bdSENP1

HygB degradation

Bacterial death
1:50 1:500 1:5000 1:50000 1:500000

F)
bdSUMO-degron$^{NER}$-HygB-bdSUMO*-ssrA + bdSENP1

HygB degradation

Bacterial death
1:50 1:500 1:5000 1:50000 1:500000